Microplastic as a vector in aquatic environment - binding of DCF on MP's and oxidative stress induction in *Aegagropila linnaei*

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Tiivistelmä - Referat – Abstract

Microplastics are widely studied subject and have raised concern towards water security worldwide but the vector effect of microplastic has not yet fully understood. In this study the ability of microplastic to attach hydrophobic organic compounds is tested with a nonsteroidal anti-inflammatory drug diclofenac. The ability to attach hydrophobic organic compounds has been proved by microplastics but not with diclofenac. Diclofenac is also causing water security threats nearby wastewater treatment plants because it is biologically active and can cause stress to the aquatic organisms even in small quantities.

The aim of this study is to see if microplastic has vector effect for the diclofenac. If microplastic retains diclofenac on its surface area it would decrease the stress factor effect of diclofenac towards the investigated macroalgae *Aegagropila linnaei.* The possible change of oxidative stress levels in *A. linnaei* is measured by peroxidase enzyme activity. The aim is to see if the enzyme activity raises or decreases when *A. linnaei* is exposed to microplastic with and without diclofenac. If the peroxidase enzyme activity decreases in macroalgae while exposing *A. linnaei* to both microplastic and diclofenac it would strengthen the vector effect hypothesis.

As a result, the peroxidase enzyme activity seems to have a decreasing trend when the diclofenac concentrations increase. Diclofenac affected to peroxidase enzyme activity but microplastic does not show any signs of binding of diclofenac in this study, and therefore microplastic cannot act as a vector for diclofenac.

Keywords

Microplastic – diclofenac – vector effect – oxidative stress – aquatic ecosystem

Säilytyspaikka - Förvaringsställe - Where deposited

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Mikromuovi vektorina akvaattisessa ympäristössä – diklofenaakin sitoutuminen mikromuovin pinnalla ja oksidatiivinen stressi *Aegagropila linnaeissa*

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Tiivistelmä - Referat – Abstract

Mikromuoveja on tutkittu hyvin laajasti, ja samalla on noussut huoli vesiturvallisuudesta maailmanlaajuisesti. Vaikka mikromuoveja on tutkittu paljon, mikromuovien toimiminen vektoreina muille yhdistelle vesiekosysteemeissä ei ole täysin tunnettu mekanismi. Tässä tutkimuksessa tutkitaan mikromuovin kykyä sitoa itseensä hydrofobista orgaanista yhdettä nimeltä diklofenaakki, joka on tulehduskipulääkeaine. Mikromuovien kykyä sitoa itseensä hydrofobisia orgaanisia yhdisteitä on tutkittu ja vuorovaikutus on todettu, mutta diklofenaakin sitoutumiskykyä mikromuovin pinnalle ei ole vielä tutkittu. Diklofenaakki on biologisesti aktiivinen yhdiste ja voi aiheuttaa jopa pieninä määrinä stressiä akvaattisille organismeille, mikä aiheuttaa uhkaa erityisesti jätevedenpuhdistamoiden läheisille vesiympäristöille ja vesiturvallisuudelle.

Tämän tutkimuksen tarkoituksena on saada selville, pystyykö mikromuovi sitoa pinnalleen diklofenaakkia eli pysytäänkö havaitsemaan ns. vektorivaikutus. Jos mikromuovi sitoisi diklofenaakin pinnalleen, se vähentäisi stressitekijän, eli tässä tapauksessa diklofenaakin, vaikutusta tutkittavaan makrolevään nimeltä *Aegagropila linnaei.* Tässä tutkimuksessa makrolevän stressitasoa kuvastaa oksidatiivisessa stressissä tapahtuvat muutokset, jotka mitataan peroksidaasientsyymiaktiivisuudella *A. linnaeissa*. Tarkoituksena on havaita, nouseeko vai laskeeko entsyymiaktiivisuus kun *A. linnaei* altistetaan mikromuoville diklofenaakin kanssa ja ilman. Jos peroksidaasientsyymiaktiivisuus laskisi makrolevässä, kun se altistettaan mikromuoville ja diklofenaakille samaan aikaan ,se vahvistaisi vektorivaikutus-hypoteesia, jolloin mikromuovi sitoisi pinnalleen diklofenaakkia.

Tutkimuksen tuloksista voidaan havaita, että peroksidaasientsyymiaktiivisuus laskee, kun diklofenaakki konsentraatiot nousevat. Diklofenaakki vaikuttaa peroksidaasientsyymiaktiivisuuteen, mutta näiden tulosten perusteella mikromuovi ei pysty sitomaan diklofenaakkia pinnalleen, eikä täten pysty toimimaan vektorina diklofenaakille.

Keywords

Mikromuovi – diklofenaakki – vektorivaikutus – oksidatiivinen stressi – akvaattinen ekosysteemi

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Content

1. Inroduction

1.1 Microplastic and its problems in the environment

Plastic waste has attracted great interest around the globe due to its harmful features in the environment. Production of plastic waste has contributed to the microplastic (MP) problem and researchers have paid attention to MPs, especially in the aquatic environment (Yu et al. 2018). In the year 2015, the annual plastic production was 380 million tons (Geyer et al. 2017). In the same year, around 55 % of the plastic was discarded 25 % was incinerated and 20 % was recycled (Geyer et al. 2017). We can see the massive gyres of plastic debris in the open sea, which can be degraded into smaller fragments (Cózar et al. 2014). When not recycled or disposed of properly, plastics are degrading into MPs and can cause great concern either as primary or secondary MP. Primary MPs are created for the purposes which need small size plastics like cosmetics or pharmaceuticals (Cole et al. 2011; Peng et al. 2017). Secondary plastics, on the other hand, are formed when greater size plastics pieces are degraded into smaller fractions by UV radiation, physical or biological means like solar radiation, abrasion or microbiological activity (Peng et al. 2017; Graca et al. 2017). Secondary MPs can be formed for example, from washing synthetic clothes, breakdown of plastic debris and car tires. Fibers from washing clothes usually go through the wastewater treatment plants where the fibers are mostly retained. The breakdown of plastic products in the environment and MP released from car tires drift straight into various water systems. Car tires can lose 10 – 20 % of its mass during its lifetime, so roads are one possible pathway for MPs to wash into water systems (Kole et al. 2017). Secondary MPs have been shown to drift into the aquatic ecosystems when plastics are degraded in water ecosystems or more significantly *via* wastewater treatment plants (Li et al. 2016; Carr et al. 2016).

MPs are defined as plastic polymer particles which size range is between 1 nm and 5 mm (Koehler et al. 2015). MP has been detected in oceans and sediments all over the planet (Thompson et al. 2004; Qiu et al. 2015; Gray et al. 2018). The ability of plastic to degrade into its monomers, either by biotic or abiotic ways, is quite week (Chamas et al. 2020) and therefore MP has many opportunities to stay in the environment and cause problems for organisms. The MPs entering food webs cause harm to the organisms by ingestion but also *via* chemical effects in the water column (Graca et al. 2017; Li et al. 2016). MP can starve microorganisms when ingested because of the lack of nutrients. The surface of MP can also act as a platform for biofilm communities which can possibly be a hazard because of the ability to transport of pathogens (Kirstein et al. 2016). The chemical effects of MP to the aquatic

environment have been studied and studies have shown that MPs can act as a vector for hydrophobic organic compounds (HOCs) such as polyaromatic hydrocarbons, bisphenol A, PCBs and other organic chemicals (Liu et al. 2017; Velzeboer et al. 2014).

MPs effects on chemical compounds such as HOCs have been studied and the possible reducing or increasing negative factors facing aquatic organisms has been extensively discussed. Rehse et al. (2018) has studied the ability of bisphenol A (BPA) to attach on polyamide particles (PA) and how zooplankton is reacting to that. In the Rehse et al. (2018) study PA reduced the effect of BPA on the zooplankton and the same kind of reaction was expected to see in this study. As well, MP has been tested with other types of pharmaceuticals and the sorption percentage was calculated and the sorption rates varied between 15.31 – 28.61 % respectively (Razanajatovo et al. 2018). The short-term toxic effect of HOCs such as PAHs has been recorded to decrease when exposed to the bacterial community together with MP (Kleinteich et al. 2018).

The surface of MPs is hydrophobic so the chemical compounds which are also hydrophobic could have the ability to attach on the surface of the MP in aquatic circumstances (Anderson et al. 2016). Also, MP itself can contain potentially harmful chemicals, additives, and when leached out, they can cause a potential threat to the aquatic organisms (Teuten et al. 2009; Hahladakis et al. 2018). It has been studied that different types of plastic adsorb the HOCs in different intensities (Liu et al. 2019; Lee et al. 2014). In this study, the used MP is polyethylyne (PE) and it is tested with the HOC diclofenac (DCF).

Marine ecosystems with MP problem are better documented than freshwater systems, but the threat also exists in freshwaters and towards aquatic organism as well. The fate of MP has been well documented by Li et al. (2016) and in many situations, what happens in the sea can also happen in freshwaters. MPs are detected in freshwater systems in USA, Europe, UK and Asia but the studies of MPs in freshwater are still quite an unknown research area and yet important (Eerkes-Medrano et al. 2015; Horton et al. 2017). There are proves that MPs exist even in the most remote areas like Tibetan lakes in China (Zhang et al. 2016). Su et al. (2016) discovered that MP concentrations range from 3.4 to 25.8 items L^{-1} in surface water of Taihu Lake, which is the third largest lake in China and reportedly affected by human activity.

As mentioned earlier, the pathways for MP into freshwater systems can vary but the main source in most cases is wastewater treatment plants (WWTPs) (Li et al. 2016). Results show that 95 – 99 % of

incoming MP can be retained into the sludge of wastewater treatment (Talvitie et al. 2017). Even though the percentage of retained MPs is very high, the amount of leached MP particles can be up to 65 million pieces per day, which makes it quite significant in the long run (Peng et al. 2017). The amount of leaked MP from wastewater treatment plants WWTP, breakdown of plastic products on land and in the sea creates together a threat to aquatic environments. It is also important to note that freshwater systems like rivers can be one of the major transportation pathways of MPs into the ocean. That is one reason why it is necessary to investigate freshwater ecosystems and see the link between the ocean and freshwater MP load (Peng et al. 2017).

MPs are a widely studied topic these days because of the rising concern of MPs' harmful effects towards organisms in the environment and through the food chain possible threat to humans too. MPs are part of the plastic waste problem, but it is not the only problem. If trusting the current trend of productions rate of plastic, the plastic productions will not decrease in the near future and the usage of plastics can even rise (Geyer et al. 2017). The threat of the MP problem becomes more relevant due to the increasing amount of plastic waste. Proper ways to manage plastic waste and MP waste should be invented and taken into action. If plastic waste is not removed from environment, it will face degradation and eventually end up being MP which will be almost impossible to remove from nature, or at least very challenging and resource consuming.

More investigation is needed to understand the interaction between MP and other contaminants, such as DCF in this study, and to see how strong and what type of harm they can cause together to aquatic organism. The potential risk of DCF and MP together is assessed in this study, and the concentrations, which are higher than in the environment, are used to see the possible connections between these two contaminants.

1.2 Diclofenac as a threat to the aquatic environment

Diclofenac (DCF) is a pharmaceutical compound also known as a non-steroidal anti-inflammatory drug, which is used mostly in medicine as a painkiller (EMA 2013). The most well-known medicine containing DCF is probably Voltaren which can be put onto the skin to relieve inflammation and pain. DCF ends up into the environment because of the humans and more specifically through humans. According to Zhang et al. (2008), humans worldwide consume 940 tons of DCF and all in all the pharmaceutical usage is increasing annually for example due to easier availability of medicine and

population growth (Comber et al. 2018). The alarming point of view of the consumption amount is that DCF does not entirely absorb into humans through the skin when using DCF gel. When DCF is ingested a great quantity of DCF will go through human bodies changed and unchanged and end up in wastewater treatment plants. After oral ingestion of DCF, 60 to 70 % is excreted by urine and faeces and when put onto the skin only 6 to 7 % will be absorbed into the human body (Davies & Anderson 1997).

Not only humans but also livestock are often treated with pharmaceuticals which include DCF (Sathishkumar et al. 2020). The problem with the DCF use in livestock is related to other animals like vultures (Oaks et al. 2004). The European Medicines Agency's (EMA) has stated that carcass that has been treated with medicine which includes DCF can cause kidney failure and death to vultures (EMA 2014). The need for medication (specifically DCF) has created pollution problem from point sources like wastewater treatment plants (WWTPs) and livestock farms but also non-point sources such as untreated wastewaters from domestic, industrial and agricultural activities (Sathishkumar et al. 2020).

Some of the WWTPs can remove part of DCF out of wastewater afterwards but the efficiency depends on the treatment methods (Bonnefille et al. 2018). A very common treatment method used in WWTPs is a biological treatment which has many advantages including efficient organic waste removing capacity with activated sludge and it is also cost-effective (Mittal 2011). Although DCF is partly treated in WWTPs, the removal rates of DCF vary a lot in and between WWTPs in different countries. According to Zhou et al. (2009) removal rate of DCF is between 70 – 92 % in the UK WWTPs. Zhang et al. (2008) showed that the removal efficiency could vary between 0 % and 80 % but the most usual removal efficiency was 21 – 40 % in WWTPs. The removal rate of DCF and other pharmaceuticals were studied in five EU countries and removal rate for diclofenac was only 39 % (Paxéus 2004).

WWTPs are point sources for pharmaceuticals so concentrations in the nearby water systems are often observed. Zhou et al. (2009) measured the DCF concentration from three different WWTPs in England. The DCF concentrations in WWTPS effluents measured were 119, 176 and 78 ng L^{-1}. It is important to note that even though the removal efficiencies were high (70.1, 77.5 and 92.0 %), the concentrations in effluents could be quite high because the input concentration are so substantial. Lee et al. (2019) have also compared the DCF concentrations between Gyeongsan WWTPs influents and effluents and ascertained that concentration in influent was 0.11 µg L^{-1} and in effluent 0.13 µg L^{-1}. The same kind of increase in DCF concentrations in the effluent was detected in Kristianstad in Sweden (influent: 122 mg/d/1000 inhabitants; effluent: 251 mg/d/1000 inhabitants) (Zorita et al. 2009). The concentrations of DCF can be quite high in effluents but even though the removal rates

of DCF can also be high the effluents may still contain DCF which is a threat to aquatic environment. European Guideline on the Environmental Risk Assesment of Medicinal Products for Human Use (EMA 2006) has stated the methods for calculting the risk of pharmaceuticals in the aquatic environment with measured/predicted environmental consentration (MEC/PEC) and predicted no-effect concentration (PNEC). If the risk quontient (RQ) of MEC or PEC/PNEC is greater than one there is a risk for the aquatic environment. As Sui et al. (2010) study shows that the risk of DCF for the aquatic environment was higher than one (2.04) and Bouissou-Schurtzs et al. (2014) results show that the RQ is 15 when calculation was done by using PEC/PNEC.

The DCF concentrations have also been detected from surface waters (Lonappan et al. 2016). In Spain DCF concentrations have varied between 2.8 and 46.0 ng L^{-1} with a mean concentration of 13.6 ng L^{-1} (Iglesias et al. 2014) and in Finland in Lake Päijänne the DCF concentrations are between 100 – 450 ng L^{-1} (Lindholm-Lehto et al. 2016). DCF concentration values have been measured from east and west sides from Dongting Lake in China (Ma et al. 2016). The surface water DCF concentrations are between 3.3 – 230.5 ng L^{-1} on the east side and 2.1 – 77.9. ng L^{-1} on the west side. As seen from the variation range, DCF concentrations may vary remarkably even within short distances. It is urgent to know what is the predicted no-effect concentration (PNEC) for certain compounds. PNEC tells the lowest concentration when there is no predicted harm to the organisms. There are several statements about PNECs for DCF including 50 ng L^{-1} (Comber et al. 2018), 100 ng L^{-1} (Sui et al. 2010) and 10 µg L^{-1} (Carlsson et al. 2006) but all in all the PNEC for DCF can be argued to be quite small. Sathishkumar et al. (2020) made a summary of DCF concentrations in surface waters and other environmental matrixes and they found out that the DCF concentrations were at its highest 57.16 µg L^{-1} in Nigeria which is, compared to any of the argued PNECs, relatively high.

Although the concern towards DCF leaching out to the environment has been rising, the removal of DCF has not been made mandatory in the law. DCF concentrations have been detected to be high nearby the WWTPs in water column and for example, the region of Baltic sea is getting its share of DCF because of poor removal efficiencies (HELCOM 2018). DCF was added among few other pharmaceuticals to the European Water Framework Directive (WFD) ‘watch list’ (EU, 2013) but in 2018 in JRC report DCF was suggested to be removed from that list as a result of sufficiently high-quality monitoring data (Loos et al. 2018).

Globally, DCF concentrations in the environment are a problem and its removal is well justified because DCF has detected in aquatic environment and organisms and causing them harm (Mezzelani

et al. 2018). Brozinski et al. (2013) found DCF in the fish species nearby the WWTPs downstream in Haapajärvi Finland. DCF has been detected to affect to eating habits of fishes (Nassef et al. 2010), cytopathological alterations in liver, kidney and gills (Triebskorn et al. 2004), and gene expression in *Daphnia manga* (Liu et al. 2017). Exposure to DCF only at 1 µg L^{-1} concentration resulted in reduced gill surface area through cell necrosis and precipitates on the gill surface in rainbow trout (Triebskorn et al. 2004). In Feito et al. (2011) study 0.03 µg L^{-1} of DCF induced lipid peroxidation decrease in zebrafish *Danio rerio* when acute toxicity was tested. The acute toxicity was also tested with fern *Polystichum setiferum* and fern gave acute lethal phytotoxicity at 24 h (30 µg L^{-1} DCF) and 48 h (0.3 µg L^{-1} DCF). Even though the risk for acute toxic effects has been stated to be unlikely the concern about chronic ecotoxic effects must be taken into account too (Carlsson et al. 2006). Feito et al. (2011) assessed the chronic toxicity besides of acute toxicity of DCF for fish *D. rerio* and fern *P. setiferum* and the chronic induces could be seen DNA quantification in spores after 1 week (LOEC) 0.03 µg L^{-1} DCF exposure.

As Feito et al. (2011) proved in their study, DCF is affecting to aquatic plants. *Lemna minor* and *Lemna gibb*a are affected by DCF which triggers their defense mechanism by increasing, for example, catalase (CAT) activity in oxidative stress (Alkimin et al. 2019). *L. minor* has also been detected to suffer from negative growth response and decrease of photosynthetic pigments (Kummerová et al. 2016). Alkimin et al. (2019) pointed out that even within the same genus DCF can cause different responses so the effects of DCF is dependent on the certain species. The stress effects have been detected as well in *Populus alba* L. but at the same time, the phytoremediation level and removal of DCF from wastewater were found to be successful (Pierattini et al. 2018). One indicator for oxidative stress in plants is Glutathione S-transferase (GST) activity levels which were shown to increase in *Typha latifolia* in the presence of DCF (Bartha et al. 2014). Different plant species will be affected in different ways and studies differ from what type of endpoints they are using in their research. Endpoints can vary between different enzymes like GST (glutathione S-transferase), POD (peroxidase) and CAT (catalase), in their activity but also within where the enzymes or other indicators are detected such as roots or leaves, or how the growth rate is affected (Alkimin et al. 2019; Pierattini et al. 2018; Bartha et al. 2014).

Carlsson et al. (2006) have made ecotoxicity table which covers many different types of organism and pharmaceutical ingredients. DCF exposure for *S. capricornutum* (green algae) has the lowest observed effect concentration (LOEC) 20 mg L^{-1} and *Desmodesmus subspicatus* (green algae) has the half-maximal effective concentration EC_{50} (72 h) value 72 mg L^{-1} (Ferrari et al. 2003; Cleuvers

2003). *L. minor* (vascular plant) has the EC_{50} 7.5 mg L^{-1} (DCF) in seven days (Cleuvers 2003). It can be noticed that DCF has an impact on aquatic plants like algae and that the effects of DCF depend highly on the examined species.

The degradation of DCF has been detected in the aquatic environments and the rate of degradation can be quite high, the half-life of 39 minutes (Packer et al. 2003). According to Andreozzi et al. (2003), photodegradation half-life during wintertime and higher latitudes was five days so the half-life depends on seasons and latitudes. Although the rate of degradation can be high in the environment it does not invalidate the fact that DCF is bioactive and designed to be effective in low concentrations which causes threats to the organisms. Despite of the degradation, DCF is and will be leached into the environment constantly from WWTPs causing a continuous problem (Daughton & Ternes 1999). Nowadays more people have easier access to the medicine and the population is still growing (Buser et al. 1998). DCF should be studied more because it is biologically active and can cause a risk for aquatic organisms (Bartha et al. 2014).

1.3 Oxidative stress in plants

Oxidative stress can occur when antioxidant levels of a eukaryotic cell are decreasing but also when ROSs increase in the cell (Gill & Tuteja 2010). Plants have antioxidant defense mechanisms in which superoxide dismutase (SOD) can catalyze dismutation of the superoxide ($\cdot O_2^-$) and other ROSs into oxygen and hydrogen peroxide (H_2O_2) (Deavall et al. 2012). H_2O_2 can be reduced by catalase (CAT), peroxidase (POD), ascorbate peroxidase (APx), glutathione reductase and glutathione peroxidase (GPx) (Gill & Tuteja 2010; Esterhuizen-Londt et al. 2011). Oxidative stress and the defense mechanisms can be observed from the Fig. 1. The risen amount of POD indicate the increased stress level and it is used in this study evaluate the differences between the exposures and treatments. It must keep in mind that other antioxidant pathways are not considered in this study but POD is used as a biomarker in this study. In this study oxidative stress is detected through POD activity and the total protein concentration revealed by Bradford's test (Bradford 1976).

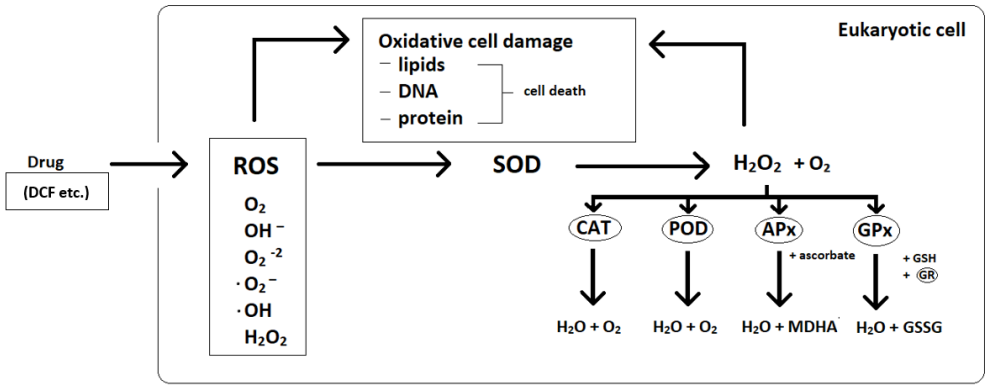

Figure 1. Oxidative stress in eukaryotic cells and antioxidant defense system against reactive oxygen species (ROS). Drugs or other toxic compounds may enter to eukaryotic cell and when converting to ROS, superoxide dismutase (SOD) transfers ROS into peroxide (H_2O_2) and oxygen (O_2). Catalase (CAT), peroxidase (POD), ascorbate peroxidase (APx) and glutathione peroxidase (GPx) reduce H_2O_2 to harmless compounds (Gill & Tuteja 2010; Esterhuizen-Londt et al. 2011; Deavall et al. 2012).

1.4 Hypothesis, research question and scenarios

The hypothesis of this study is that MP could act as a vector for DCF and prevent the oxidative stress in macroalgae. The focus of this study is on the chemical effects on the organism called *A. linnaei*, macroalgae, which will be exposed to MP and DCF.

MPs ability to attach DCF is tested in this study and the research question is: does MP attach DCF on its surface and therefore inhibit the DCF from causing oxidative stress to the test plant as DCF would by itself. In this study the oxidative stress is measured by the amount of POD which is one of the main antioxidative defense mechanism against stress factors for the plant. Under stressful circumstances organisms' level of reactive oxygen species (ROS) increases which leads to oxidative stress along the chemical process.

Three scenarios were tested: 1) MP does attach DCF on its surface and DCF stays on it; 2) MP does attach DCF on its surface but also releases DCF back into water; 3) MP does not attach DCF on its surface. MPs and DCFs effects are also measured separately so it can be known how macroalgae *A. linnaei* reacted to these compounds independently.

DCF impacts has been tested by other organisms such as daphnia, fishes and with plants *L. minor* and *L. gibba* (Alkimin et al. 2019) but the list of aquatic plants is still quite narrow. Now in this study the

effects of DCF on the macroalgae *A. linnaei* is tested with MP. This study is a novelty study because of new compound connections between MP and DCF and their effects on oxidative stress in *A. linnaei*.

1.5 The aim of the study

In this study, the aim is to combine these two topics (MP and DCF) to see if MP is a vector for DCF and if it induced oxidative stress compared to MP or DCF alone. Near by the fresh waters can be found WWTPS which can be possible pathway for both MP and DCF into the aquatic ecosystems and therefore the presence of both compounds at the same time is relevant. The hydrophobics (HOCs) have the ability to attach on MPs so MPs can act as vectors for HOCs (Liu et al. 2019). The ability of DCF to attach on MP has not yet studied and the inhibitor effect of MP for DCF is still yet unknown so in this study their binding and release of DCF will be tested *via* oxidative stress enzyme POD.

In this study the tested concentrations differ from detected concentrations of MP and DCF found in the natural environment. The recorded environmental concentrations of DCF are measured in ng or µg whereas tested DCF concentrations in this study are measured in mg. Reason for higher concentrations for compounds is that at first it must be known if there is a correlation between MP and DCF which can be even detected. If correlation between tested compounds exist, it would be proceeded with other studies with more realistic concertation found in the nature. In ecotoxicological research toxic levels are quite often criticized being too high for the natural condition (Lenz et al. 2016) but in novelty studies it can be well justified because of the need to know if there even exists correlation. The other aspect with MP besides concentration is the size and age of MP. In the environment it is uncommon to see regular size and same age MPs. In this study new, very small size (3 – 8 µm) and same shape microbeads were used to see the possible interaction between MP and DCF without any interface of other chemicals and with the greatest surface area possible.

In this study the aim is to find out does DCF bind to MP and is there reducing effect for the toxicity of DCF when bind to MP. The hypothesized binding will be measured by oxidative stress in test algae called *Aegagropila linnaei*. The more oxidative stress happens inside of the algae the less binding is happening between DCF and MP.

2. Material and Methods

2.1 Preparing of microplastic and diclofenac solutions

MP solution used in this study was prepared with tap water mixed with MP powder and the solutions concentration was in the end 0.5 g L^{-1}. MP used in this research is polyethylene (PE) round shape beads and the average size between 3 to 8 µm. DCF used in this study was diclofenac sodium ($C_{14}H_{10}Cl_2NNaO_2$) (CAS 15307-79-6) which means that the DCF was not pure diclofenac. DCF solutions were prepared in three different concentrations 0.5 mg L^{-1}, 1.0 mg L^{-1} and 5.0 mg L^{-1}. *A. linnaei* was exposed only to MP (0.5 g L^{-1}) in the first treastment and in the second treatment *A. linnaei* was exposed to DCF in three different concentrations (0.5 mg L^{-1}, 1.0 mg L^{-1} and 5.0 mg L^{-1}). When exposing the macroalgae only to MP the idea was to verify if there were any leaching additives from MP which could had affected to *A. linnaei*. The second treatment with only DCF showed how *A. linnaei* would respond to DCF as a stress factor. In the third treatment MP was mixed with three different DCF concentrations (0.5 mg L^{-1}, 1.0 mg L^{-1} and 5.0 mg L^{-1}) and the *A. linnaei* was exposed to the mixture. With the third treatment it could hypothetically be shown if the stress levels (POD) would increase or decrease compared to the second treatment which has only DCF. The fourth treatment was accomplished also with MP and DCF together, but the mixed solutions with three different concentrations of DCF (0.5 mg L^{-1}, 1.0 mg L^{-1} and 5.0 mg L^{-1}) were filtered out of MP before exposing *A. linnaei* to eluent. The eluent treatments were executed to see if the scenario number two (2) could happen and could MP retain DCF on its surface which would reduce the stress factor effect or the other way around. All the prepared solutions were stirred 48 h in measuring bottles which were wrapped in foil. *A. linnaeis* were exposed to treatment solutions in glass beakers for five (5) days after stirring.

2.2 The examined macroalgae *Aegagropila linnaei*

The test plant used in this study was *A. linnaei* which is a multicellular freshwater macroalgae and naturally found in central and northern Europe but also widely in Japan (Boedeker et al. 2010). The ball-shaped form of *A. linnaei* was used in this study to see the effects of the contaminant DCF in the water in laboratory conditions. Algae species are often studied how do they response to contaminants in the water (Hamed et al. 2017). The ability to uptake the contaminants such as cyanotoxins BMAA and MC-LR from water media have been proven with *A. linnaei* (Contardo-Jara et al. 2015). The

oxidative stress effect is one way to measure the effects of contaminations in algae (Hamed et al. 2017). Therefore in this study *A. linnaei* was tested with pharmaceutical DCF to see the possible oxidative stress reaction through POD enzyme activity.

2.3 The chemical exposure with and without microplastic

In the first scenario DCF could attach on the surface of the MP. In the second scenario DCF can attach on the MP, but MP can also release DCF back into the solution. In the third scenario DCF does not attach on the MP at all. To test these three scenarios *A. linnaei* were exposed to prepared solutions: MP and DCF alone, together and filtered eluent which included only DCF after the MP was filtered out of the solution. The exposure time for the *A. linnaei* was five days under the laboratory conditions. Each treatment and concentration had five replicates and five controls.

2.4 The extraction of the peroxidase enzymes

After the exposure time T_5 (five days) moss balls were wrapped in foil and stored at – 80 °C to stop the enzyme activity. The exact moment of peroxidase (POD) enzyme activity were captured when the moss balls were frozen. Frozen moss balls were crushed into powder by using a pestle, a mortar and liquid nitrogen to keep the samples frozen during the whole procedure. Crushing samples into fine powder was crucial to gain extracted POD enzyme as much as possible.

After the moss ball samples were crushed, the enzyme extraction was proceeded via S9 fraction protocol. Moss ball samples were put 1 g in the beakers with 2 mL of 20 mM Cytosolic NaP buffer which pH 7.0 was mimicking the natural environment of the cell. The samples were kept under 4 °C during the extraction which protected the POD enzymes from breaking down by proteases. Samples were stirred 20 min with a magnetic stirrer and then put into the centrifuge with 9.000 g for another 20 min. Centrifugation separated algae debris from enzymes and the supernatant fraction of the samples with POD enzymes were put back in the freezer – 80 °C.

2.5 Bradfords protein determination

Before the enzyme activity could be estimated the total protein concentration of the samples had to accomplish. In this study the protein determination was done by using Bradfords protein determination test and Bradfords reagent (Bradford 1976). The protein determination was based on Coomasie Brilliant Blue G-250 binding on the protein which meant that Coomasie Brilliant Blue G-250 could bind on any present protein in the sample solution. When the binding happened on the protein the absorbance changes from 465 nm to 595 nm and the change was measured with spectrophotometric analytical device (Tecan plate reader). BSA standard curve was prepared using concentrations: blank, 5, 10, 50, 100, 250 and 500 µg mL^{-1}. The total protein concentration was calculated for each sample by the help of BSA standard curve. The greater the absorbance change was the more proteins there were in the sample solution.

2.6 Peroxidase enzyme activity analysis

Increase of the peroxidase enzyme activity level is caused by the oxidative stress in plants (Fig. 1). When the plant confronts a stress factor around its environment this causes formation of reactive oxygen species (ROS) which can lead damages to the cells. To avoid the damages (eukaryotic) cell have enzymes called superoxidase dismutase (SOD) which convert radicals into oxygen (O_2) or hydrogen peroxide (H_2O_2) in the cell. Hydrogen peroxide will also cause problems inside the cell which can be inhibited with catalase (CAT), peroxidase (POD), ascorbate peroxidase (APx) or glutathione peroxidase (GPx). Peroxides are one of the common ROSs so the levels of the peroxides increase when stress factors are present in organisms and at the same time the level of the peroxidase enzymes will also increase to prevent the damages of the peroxides. The amount of PODs were quantified in this study through the peroxidase enzyme activity assay.

The activity of peroxidase was measured by using guaiacol and peroxide. In the chemical reaction the peroxidase was the catalytic enzyme which fastened the reaction in which H_2O_2 was reduced to water and guaiacol was oxidized into quinone (Fig. 2.). The higher intensity of quinone the higher quantity of peroxidase.

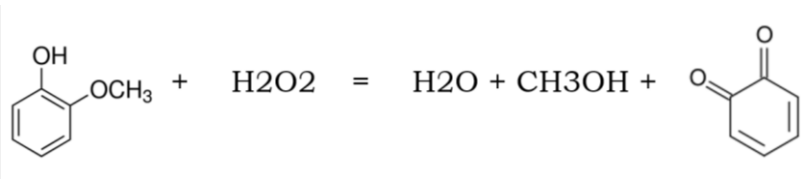

Figure 2. The oxidation of guaiacol to quinone.

H_2O_2 reagent was prepared by measuring 100 µL of H_2O_2 (30 %) in 4.9 mL NaP (20 mM, pH 7) buffer. Guaiacol reagent was prepared by measuring 0.5 mL guaiacol in 4.5 mL DMSO. These two reagents were prepared in glass beakers. The reagents used were 200 mM H_2O_2 and 100 mM Guaiacol solution.

The measured activity indicates how many catalysis happened in mg of protein when one catalysis meant that one mole of substrate was catalyzed per one second (Katal = 1 mol/s). In this study mKatal indicated how many mmol of substrate was catalyzed in one second so how many guaiacol molecules were oxidized to quinone while H_2O_2 was reduced to water. Peroxide was added 10 µL as a substrate to all samples during the peroxidase enzyme (POD) activity assay to test the catalysis reaction of peroxidase enzyme. The more there were peroxidase enzymes the faster the reaction was. The peroxidase enzyme activity was calculated by using the formula:

$$AE = \frac{\Delta E \times VW}{Vp \times \varepsilon \times d \times cprop \times \Delta t}$$

Where AE is enzyme activity, ΔE stands for change in absorbtion, VW stands for total volume pipetted in the tecan plate column, Vp in the sample volume pipetted, ε is the extinctions coffiecient, d refers to light path in the column, $cprop$ is the total protein concentration, and Δt change in time when the acitivity was measured.

2.7 Preparing DCF samples for UPLC – MS/MS analysis

The exact concentrations of DCF of each solution were analyzed by UPLC – MS/MS and the water samples were taken right before adding MP into the solution, after 48 h stirring, after filtration MP out of the solution and after 5 days exposure to the moss balls. The amount of DCF to attach onto MP and the possible photodegradation were estimated *via* this analysis method. The samples were diluted

from 0.5 mg L^{-1} to 0.05 mg L^{-1}, 1.0 mg L^{-1} to 0.1 mg L^{-1} and 5.0 mg L^{-1} to 0.5 mg L^{-1} because otherwise the concentrations would have been too high for the UPLC devise to analyze them. After dilutions the samples were filtered *via* GHP Acrodisc ® 13 mm Syringe Filter with 0.45 µm GHP Membrane into gas chromatogram vials. These gas chromatogram vials were put into the UHPLC-MS/MS analysis device.

2.8 Diclofenac determination from exposure solution via UHPLC – MS/MS

The UHPLC – MS/MS analysis of DCF was completed by using AQUITY UPLC ™ (Waters, Manchester, UK) instrument which had Quatro premier tandem quadrupole mass spectrometer (Waters). The chromatogram peaks were measured from negative side of electrospray ionization (ESI) and separation of the DCF was completed by using ACQUITY UPLC® BEH C18 (2.1 mm x 50 mm, 1.7 µm particle size). In gradient mobile phase it was used 5 % methanol (eluent A1) and 100 % methanol with 1 mM ammonium fluoride (eluent B1). Gradient conditions were 0.00 – 2.50 min, 50 % B1; 2.50 – 10.10 min, 100 % B1, 10.10 – 12.00 min, 50 % B1 in which flow rate was 0.2 mL min^{-1} and the injection volume was 5 µL. Total run time for one sample was 9.50 min. The instrument parameters were as follows in Table 1.

Table 1. The instrument parameters of ion source and multiple reaction monitoring.

Ion Mode	Electrospray $^-$	
Capillary voltage (kV)	3	
Desolvation temperature (°C)	300	
Source temperature (°C)	120	
Collision gas	Argon	
Desolvation gas flow (l h^{-1})	700	
Cone gas flow (l h^{-1})	50	
CV/CE (V/eV)	15/15	
MRM parameters	1	2
Parent ion (m/z)	293.8	293.8
Daughter ion (m/z)	212.1	249.9

3. Results and Discussion

3.1 DCF determination with UPLC-MS/MS

The lowest concentration of diclofenac (DCF) (0.5 mg L^{-1}) were rerun with UPLC-MS/MS after all samples were analyzed. The results of the lowest concentration treatment (0.5 mg L^{-1}) showed that the real concentration in all samples would have been around 3 mg L^{-1}. The samples were diluted 1:10 at first and the results showed concentrations 0.3 mg L^{-1} so when the concentrations were multiplied with 10 the real concentrations seemed to be around 3 mg L^{-1}. The quantity of DCF weighted was quite small (5 mg) when the stock solution was prepared. The smaller amounts weighted on the scale the greater the risk is to have measure errors. In this case that was most likely what happened so that is why the assumed concentrations did not match with the real concentrations. This part could be redone by making more concentrated stock solutions where DCF concentrations would be higher and then dilute those solutions into prefered concentrations. There was also a possibility that the solutions in the actual treatments had a greater quantities of DCF and so the POD activities might had been affected more strongly than expected.

Although the determined concentrations of samples in the lowest concentration of DCF (0.5 mg L^{-1}) via UPLC-MS/MS differed from the concentration what was weighted in the stock solutions it can be seen that all DCF concentrations were staying at the same level during the treatment process (5 days). There was no significant difference within any of the examined concentration groups (0.5, 1.0, 5.0 mg L^{-1}) during the treatment (Fig. 3). Staying at the same DCF concentration level during the whole exposure treatment could mean that DCF was not degrading during the process. Stable DCF concentrations might also mean that DCF was not attached on the surface of MP (0.5 g L^{-1}) or not at least significantly when comparing the DCF concentrations in the solutions and in the eluents. If the DCF concentration stays in the water column at the same level it may not be attached on the MP surface or it can also be released right away if attached. This type of reversible method was not examined in this study.

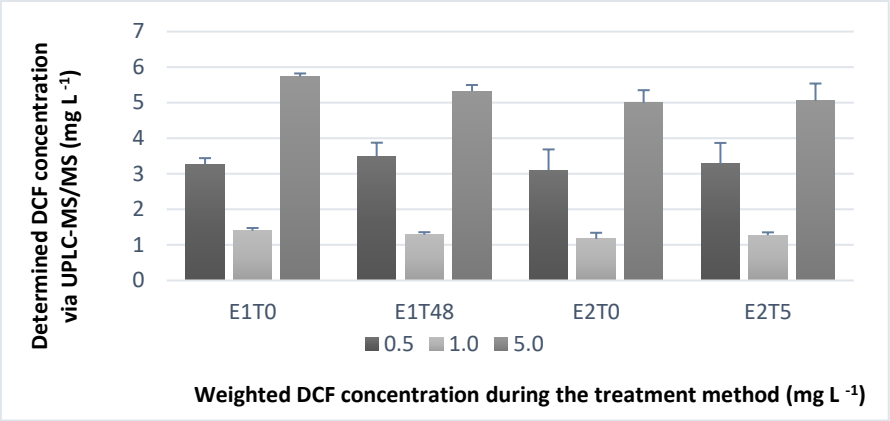

Figure 3. Determination of DCF concentrations during the exposure time via UPLC-MS/MS. Measuring points of DCF concentrations were E1T0 = before adding MP into DCF solution, E1T48 = after adding MP and stirring the mixture solution of MP and DCF for 48 h, E2T0 = after filtrating MP out of the mixture solution, and E2T5 = after five days exposure of eluent (DCF) with *A. Linnaei*.

In this study DCF was present in water column which means that DCF was available for the macroalgae, *A. linnaei*, and DCF had the ability to cause oxidative stress for the algae. There were no negative controls made for this DCF concentration examination but positive control (standard curve) were done successfully for UPLC-MS/MS run (R^2 = 0.999). This step was proceeded after the samples for enzyme activity test was done which means that concentrations between these experiments might vary. The samples for both tests (DCF analysis and enzyme activity) could be taken from the same treatment solutions which could be one improvement for this study protocol.

3.2 Peroxidase enzyme activity and oxidative stress

In this study peroxidase (POD) enzyme activity reflected the oxidative stress in *A. linnaei* which was caused by pharmaceutical DCF. Algal species have been used previously to detect to harmful effects of pharmaceuticals such as DCF but other end points like chlorophyll and number of cells have been used in other studies (Brain et al. 2007). Pharmaceuticals have the ability the cause oxidative stress in plants (Pierattini et al. 2018) and this same theory was tested for *A. linnaei* with POD enzyme activity in this study. This study was the first kind which tested how oxidative stress was induced in macroalgae *A. linnaei* in circumstances where DCF performed as a stress factor with MP.

The samples contained certain amount of peroxidase enzymes after the treatment period depending on how stressful circumstances macroalgae had. The amount of the peroxidases in the end of the treatment method was examined by peroxidase enzyme activity assay.

The POD activity concentration of control group was the normal state of the peroxide concentration of the macroalgae when no stress factor was involved (Fig. 4). When the mean of the control group was compared to the other means of treatments, the statistically significant differences were found in the means of DCF 0.5 mg L^{-1} ($P = 0.02$) and eluent 0.5 mg L^{-1} ($P = 0.04$). The very high concentration of POD activity of DCF 0.5 mg L^{-1} treatment captured might signal the exact time when SOD had converted ROS to H_2O_2 and peroxidase enzyme activity increased to its highest peak.

The POD activity (mKat x mg $protein^{-1}$) was measured within all the treatments. The high concentration of POD enzyme (128 mKat mg $^{-1}$ protein, mean + SE) was detected in the lowest DCF concentration treatment (0.5 mg L^{-1}). The great difference between other treatment methods and high standard error (SE) with 0.5 mg L^{-1} DCF treatment might be result of low number of replicates so the real circumstances and reaction of the macroalgae stayed uncertain. Because of the huge variation between replicate values the two of the highest concentrations, which were not align with other values, were excluded from the examination in the Fig. 4. (C). The variations between samples could be explained also with instrumental errors but also the weighted amounts of DCF on the scale were very low (≤ 5 mg). Low weighted amounts of DCF might have affected to the accuracy of concentrations. The DCF concentrations measurements were made separately with UPLC- MS/MS so the real DCF concentrations in the treatments were not known and therefore there might be greater amount of DCF in the treatments. The higher amount of weighted DCF could had caused higher oxidative stress than expected and that could be one possible explanation why DCF (0.5 mg L^{-1}) gives so high POD acvity.

On the other hand, it might be that the concentrations of DCF (0.5 mg L^{-1}) were correct and POD activity increased when exposing the macroalgae to the lowest concentration of DCF. One explanation for that could be that the exact moment had been captured when the H_2O_2 was formed inside of the macroalgae and due to that the POD levels increased. As Feito et al. (2011) found out that DCF could have the highest impact when the concentrations were the lowest due to hormetic effect. The hormesis is debated topic but surely should be taken into account when considering results from the field of toxicology (Calabrese 2008). The lowest concentration of DCF might be affecting to the macroalgae slower than in the higher concentrations so the formation of H_2O_2 might had had delay and that was why the amount of POD activity suddenly rose enormously at the lowest concentration after exposure time (5 days).

There was statistically significant difference between control group and eluent 0.5 mg L^{-1} treatment ($P= 0.04$) (Fig. 4 (E)). The lowest concentrations of DCF (0.5 mg L^{-1}) in the treatments caused the decreasing trend of POD activity as the highest concentrations of DCF (5.0 mg L^{-1}). The tested DCF

concentrations in this study were relatively high considering the studies which had shown the risen enzyme activity in *L. minor* when exposed to 100 µg L^{-1} of DCF (Alkimin et al. 2019). In this study even the lowest concentration gave the strongest response and that is maybe one reason why the difference between lower and higher concentrations of DCF cannot be seen clearly. It would be speculated if the response could have been seen if the concentrations could have been lower.

The POD activity in eluent treatments 0.5 mg L^{-1} (DCF) were significantly lower than in control group which means that the POD and H_2O_2 levels in macroalgae were lower than the macroalgae would have in the normal state. Eluent treatment included only DCF because the MP was filtered out before exposing macroalgae to the eluent. As mentioned earlier DCF concentration did not decrease during the treatment process or after filtration so DCF was available for the macroalgae to be absorbed (Fig. 3). The POD levels were low in eluent treatment 0.5 mg L^{-1} (DCF) compared to control group after the exposure time which could reflect from already happened enzyme activity with POD enzyme activity or other protection mechanisms with other types of oxidative stress enzymes (CAT, APx, GPx). Plants have several ways to defense itself against external stress factor such as drugs which could cause oxidative stress inside of the plants (Fig. 1). The lowered POD activity concentrations might signal from already happened defense mechanism such as CAT enzyme activity which also can reduce H_2O_2 to water. The activated defense mechanisms could have lowered the H_2O_2 concentrations, and POD activity as well, significantly compering eluent treatments to control group before the exposure time was over. CAT activity were not detected in this study.

At the first sight it might seem that there is no oxidative stress happening inside of the eukaryotic cells in *A. Linnaei* and more like vice versa (Fig. 4, D). The POD activity levels were expected to increase but instead they decreased specifically in the case of eluent treatment. The sights of oxidative stress were predicted to rise POD activity concentration as the DCF concentrations rise. Hamed et al. (2017) recorded risen enzyme activity concentrations after seven days when microalgae were exposed to copper. In this study risen concentrations of POD were not recorded during the experiment period constantly but when considering about all possible pathways to lower the H_2O_2 concentration it is more likely that some type of defense mechanism had already happened which lowered the H_2O_2 concentration in five days. The POD concentrations might also vary along the exposure treatment which were not recorded in this study. Zhang et al. (2014) measured CAT, POD and SOD concentrations in *Limonium sinense* during the exposure time (7 days) when vascular plants were exposed to NaCl stress factor. It could be seen from the concentrations that in beginning enzyme activity increased and after seven days enzyme concentrations levels were even lower than in the beginning. Although the treatment plant (*L. sinense*) differed from macroalgae, *A. linnaei*, which was

examined in this study, the oxidative defense system are comparable and managed with the same antioxidant enzymes. One possible explanation for the significantly lower POD levels could indicate about previous defense mechanisms along the exposure time.

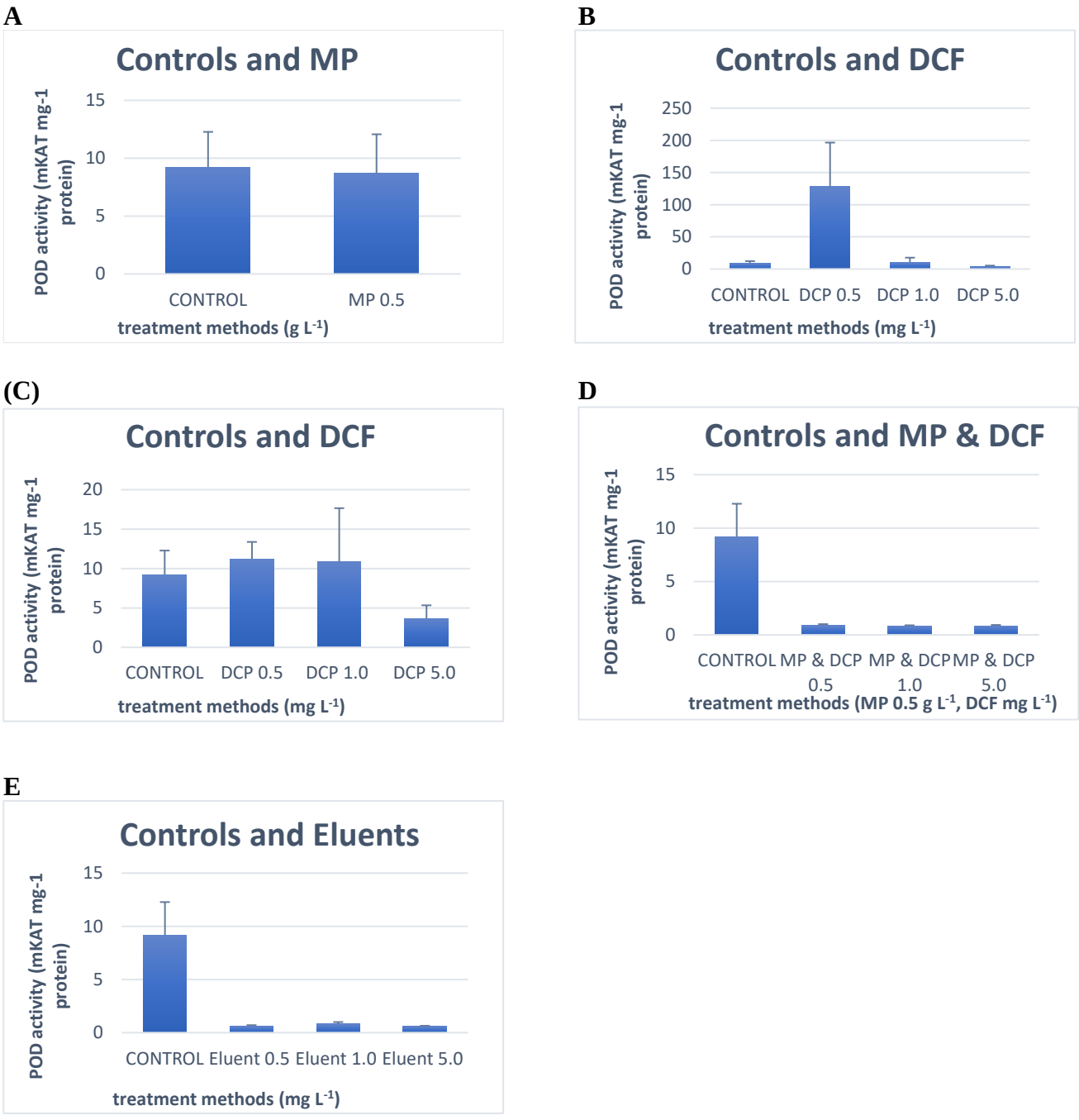

Figure 4. Peroxidase enzyme activity in treatment methods (A-E). Controls and DCF treatment were shown in two different diagrams and the other one describes the situation if the two highest values of replicates were excluded from the analyzed data (C).

There were no significant differences between the control group and MP treatment (Fig. 4, A). MP treatment was performed to see if there were any leaching detergents from the MP which could have caused oxidative stress to the treatment plant. MP treatment did not increase or decrease the POD activity concentration significantly so it can be said that MP alone did not trigger any defense mechanism and therefore did not cause oxidative stress during the exposure time to the treatment plant.

3.3 Microplastic concentration, features and size

The used concentrations for MP were fairly high (0.5 g L^{-1}) in this study to see the possible connection between MP and DCF. The concentrations of MP are occasionally high in the environment (Ma et al. 2016; Horton et al. 2017; Zhang et al. 2016) and by itself, MP can be hazardous factor in the ecosystems. The ability to attach HOCs has been proved by studies but the mixtures of MP and DCF compounds has not been investigated before and according to these results no attachment of DCF was recorded by MP. The causes of coeffect stays still uncertain. The knowledge gap stays open between DCF and MP but other types of pharmaceuticals have been studied with MP. Prata et al. (2018) studied that microplastics-procainamide and microplastics-doxycycline mixtures had greater toxic effects on algae than the pharmaceuticals alone. It has also been studied that MP can reduce the effects of HOCs such as bisphenol A and PAHs (Rehse et al 2018; Kleinteich et al. 2018). In this study the hypothesis was that MP could act as vector for DCF and reduce the harmful effects of the pharmaceutical to the macroalgae. The vector effect was not proved by these results.

MP size did not match perfectly with the size which could be found in the environment. MP used in this study was small size (3 – 8 µm) and in the environment it would take a long time for plastic to be degraded into MP that small. MP which size would be in micrometers would most likely to be absorbed or ingested by an organism before that microscopic size would be reached. The MP could have been processed in the laboratory from greater plastic into smaller size (~ 500 µm) as in Lee et al. (2014) study. It would had given more specific picture of MP in the environment. On the other hand, the interaction between MP and DCF have never been investigated so the smallest MP size could provide the greatest surface possible for DCF to attach. If there had been strong vector effect, then further investigation would have been needed with greater MP size. The concentrations used in this study are higher than naturally occurred concentration in the nature but the aim was to see the possible interaction between MP and DCF so that is why the concentrations both MP and DCF, were put higher than in natural habitat.

4. Conclusion

Unless plastic waste is not removed from aquatic environments, the amount of MP will increase in aquatic ecosystems and can cause greater threat to the organisms living in the ecosystem. Understanding the threat will increase the concerns toward the MPs and could be the accelerating factor focusing on cleaning the aquatic environments from plastic. The ability of MP to attach chemical compounds on its surface is proved by studies but its relevance due to chemical fate in the food web is still argued because of the mismatch with the concentrations in real environment and laboratory experiences, the actual sizes and the relevance of increasing or decreasing toxic effect (Lehtiniemi et al. 2018; Koelmans et al. 2016). As Koelmans et al. (2016) stated in their review HOCs were more bound to the other media in the ocean such as water, dissolved organic carbon and colloids than MP.

The WWTPs can be seen as a point source for both of the studied contaminants MP and DCF. The secondary production of MP is created by washing clothes, but also personal care products increase the MP pollution problem in the surface waters. DCF end up in the WWTPs after human use, from hospital wastewaters and also pharmaceutical industry. It is possible for these two compounds to interact in the freshwaters. These two compounds have their negative impact in the environment and therefore the interest towards MP and DCF has risen. The focus of this study was to to detect the potential vector effect of MP: do the MP decrease the exposure of DCF into the organisms. The results of this study states that MP could not act as a vector for DCF and does not have a reducing nor increasing effect on oxidative stress in macroalgae.

Feito et al. (2011) noticed possible hormesis in their study with acute and chronic toxicity of diclofenac for fern. It is possible that hormesis happened in this study. Hormesis could have seen as a higher response of POD for lowest DCF concentrations. On the other hand the high POD response for the lowest DCF concentration might be due to the error with the weighting or that the lowest used DCF concentrations gave the strongest impact possible. The stock solutions were prepared only with the latter examination of DCF concentration from the eluent treatments. Although the weighting might have some uncertainties with these results, the hormesis can be considered.

The further research with the DCF and MP compounds would include the observations in between the treatment process. Temporal examination would perceive more about the progress of enzyme activity concentrations between the starting and ending point. The results showed the end situation, but the temporal progress of POD enzyme activity stayed concealed. Besides of the temporal

examination, it would be interesting to observe other end point like CAT enzyme activity when exposing *A. linnaei* to DCF. Other aspect with improving the study between MP and DCF would be using more realistic concentrations of DCF and MP, size and origin of the MP and taking the DCF concentrations samples for the determination analysis from the same treatment solutions as the POD enzyme activity samples.

Besides the evidence that MP can intensify the effects of environmental contaminants (Prata et al. 2018) there are also proves that MP could reduce the effects of pollutant on zooplankton in the freshwater (Rehse et al. 2018). Either way, sorption and desorption on HOCs have been proven on different organisms, but the relevance of the vector effect is still under discussion. The vector effect of MP is not seen as the main pathway to the organism like macroalgae when considering the critical evaluation of all the possible media where contaminants might attach. As Koelmans et al. (2016) stated the water column itself seems to be the main pollution pathway for DCF to have an impact. In this study, DCF effected to the macroalgae but the results did not support the hypothesis where MP had reducing vector effect to DCF compound, so the hypothesis was disproved.

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